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Structural Organization of the Genes for Murine and Human Leukemia Inhibitory Factor

EVOLUTIONARY CONSERVATION OF CODING AND NON-CODING REGIONS*

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Leukemia inhibitory factor, LIF, is a glycoprotein with multiple activities in both the adult and the embryo. LIF appears to be encoded by a unique gene in both mouse and man, although the 3′-untranslated region of the mouse LIF gene gives a complex hybridization pattern on Southern blots. The complete nucleotide sequences of both the murine and human LIF genes and their flanking regions (8.7 and 7.6 kilobase pairs, respectively) were determined and compared. Both genes comprise three exons, two introns and an unusually long 3′-untranslated region (3.2 kilobase pairs), specificying a mRNA of approximately 4.1 kilobases. Two start sites of LIF-transcription were determined, by S1-nuclease protection and by a novel approach involving the polymerase chain reaction. S1-nuclease protection revealed a start site 60–64 base pairs upstream of the translational start codon and immediately downstream of a TATA box (TATATAAAT). The PCR approach identified a second transcriptional start site 160 base pairs 5′ of the start codon and adjacent to a "TATA-like" element (CATAATTT). A comparison of the murine and human LIF gene sequences revealed a high degree of conservation in the coding regions and in segments of the untranslated and flanking regions. Seven segments displaying greater than 75% homology were identified, with the 5′ and 3′ ends of the transcription unit revealing the highest degree of homology. These conserved regions represents potential *cis*-acting control elements.

Leukemia inhibitory factor (LIF)[1] is a secreted glycoprotein with diverse effects on the growth and differentiation of various cell types. It was originally purified, characterized, and cloned by virtue of its ability to induce the differentiation and suppress the clonogenicity of the murine myeloid leukemic cell line M1 (Tomida *et al.*, 1984; Gearing *et al.*, 1987; Hilton *et al.*, 1988a, 1988b; Metcalf *et al.*, 1988). More recently, LIF has been shown to suppress also the clonogenicity of the human leukemic cell lines HL-60 and U937 (Maekawa and Metcalf, 1989). Within the hemopoietic system, LIF receptors are present primarily on cells of the monocyte-macrophage lineage (Hilton *et al.*, 1988c) although their functional significance remains unclear. However, along with certain other factors active on myeloid cells (*e.g.* interleukin 6, G-CSF, and M-CSF), LIF is rapidly released into the serum of mice treated with lipopolysaccharide (Metcalf, 1988) suggesting a role for LIF in modulating an acute inflammatory response.

Although initially characterized by its differentiation-inducing effect on myeloid leukemic cells, LIF also displays a number of other biological actions. Interestingly, LIF has a potent differentiation-inhibiting activity on embryonic stem cells (Williams *et al.*, 1988; Smith *et al.*, 1988), suggesting a role for LIF in embryogenesis. Furthermore, LIF also promotes bone resorption *in vitro* (Abe *et al.*, 1986) and mice in which high levels of LIF have been experimentally achieved, display cachexia, excess new bone formation, and calcium deposition in various tissues (Metcalf and Gearing, 1989). LIF has recently also been shown to be identical to hepatocyte-stimulating factor III, a glycoprotein stimulating the synthesis of several acute phase plasma proteins (Baumann and Wong, 1989), the melanoma-derived lipoprotein lipase inhibitor (Mori *et al.*, 1989) and cholinergic neuronal differentiation factor from heart cells (Yamamori *et al.*, 1989). Taken together, these results indicate that LIF is a pleiotropic factor able to act in a variety of tissues in both the adult and the embryo.

In order to facilitate studies on the regulation and expression of LIF, we have cloned and sequenced the murine and human LIF genes. In this report we describe the genomic organization and complete nucleotide sequences of both genes and delineate the transcription unit of the murine gene. A comparison of the sequences reveals that the coding regions are highly conserved. While the non-coding regions are less related, a number of highly conserved sequences, which are candidates for *cis*-acting control elements, are evident.

MATERIALS AND METHODS

Northern Blots—Cytoplasmic polyadenylated RNA (approximately 1.5 μg), prepared essentially as described (Gough, 1983, 1988) was fractionated on 1% agarose gels containing 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA (pH 7.0), plus 6% (v/v) formaldehyde, and transferred to nitrocellulose. Prior to hybridization, filters containing RNA were soaked in 2 × SSC (where SSC is standard sodium citrate) containing 0.2% Ficoll, 0.2% polyvinylpyrollidone, 0.2% bovine serum albumin, 2 mM sodium pyrophosphate, 1 mM ATP, 50 μg/ml denatured salmon sperm DNA, and 50 μg/ml *Escherichia coli*

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05435 and J05436.

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[1] The abbreviations used are: LIF, leukemia inhibitory factor; kb, kilobase; bp, base pair; MOPS, 4-morpholineethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; PCR, polymerase chain reaction; CSF, colony stimulating factor; G-CSF, granulocyte-CSF; GM-CSF, granulocyte/macrophage-CSF; M-CSF, macrophage-CSF.

tRNA at 67 °C for several hours. Hybridization was in the same buffer plus 0.1% sodium dodecyl sulfate at 67 °C. The mouse LIF hybridization probe was the gel-purified insert of cDNA clone pLIFmut1 (Gearing *et al.*, 1987), radiolabeled to a specific activity of approximately 4×10^8 cpm/μg by nick translation and included in the hybridization at approximately 2×10^7 cpm/ml. Filters were washed extensively 2 × SSC, 0.1% sodium dodecyl sulfate at 67 °C and finally in 0.2 × SSC at 67 °C prior to autoradiography.

Southern Blots—High molecular weight genomic DNA (10–15-μg aliquots), digested to completion with various restriction endonucleases and fractionated on 0.8% agarose gels was transferred to nitrocellulose. Hybridization and washing conditions were as for Northern blots. The hybridization probes used were the gel-purified inserts of cDNA clones pLIFmut1 (Gearing *et al.*, 1987) and pLIFNK3 (Gearing *et al.*, 1988).

Library Screening—The cloning of the human LIF gene has been described previously (Gough *et al.*, 1988). For the murine LIF gene, phage plaques comprising a "Sau3A partial" genomic library from the murine plasmacytoma ABPC-17 (Corcoran *et al.*, 1985) in the λ-phage EMBL3-A (the generous gift of Dr. Lynn Corcoran, Whitehead Institute, Cambridge, MA) were grown at a density of approximately 50,000 plaques/10-cm Petri dish, transferred to duplicate nitrocellulose filters, and probed with the gel-purified insert of cDNA clone pLIFmut1 (Gearing *et al.*, 1987). Hybridization and washing were as described above except that the probe was included at approximately 2×10^6 cpm/ml.

DNA Sequencing—The nucleotide sequence of the murine and human LIF genes was determined by the chain termination method (Sanger *et al.*, 1977) using Sequenase reagents (United States Biochemicals) and [α-^{35}S]dATP (Amersham Corp.). DNA fragments to be sequenced were subcloned into the single-stranded vectors M13 (Messing *et al.*, 1981) or pEMBL8 (Dente *et al.*, 1983) and sequence determined using both "universal" primers located within the cloning vectors and primers internal to the cloned fragments synthesized on an Applied Biosystems 380A DNA synthesizer. Reactions were fractionated on 8 and 5% polyacrylamide/urea gels.

S1-nuclease Protection Assay—S1-nuclease protection analysis of DNA/RNA hybrids was performed essentially as described previously (Troutt and Lee, 1989). Briefly, total cytoplasmic RNA was hybridized with a ^{32}P-end-labeled probe (approximately 10^6-10^7 cpm/μg, 5×10^4 cpm/sample) in a 15-μl reaction containing 80% formamide, 0.4 M NaCl, 80 mM PIPES (pH 6.4), 1 mM EDTA, for 12–16 h. Hybridizations were initially performed at various temperatures between 45 and 55 °C, but then found to be optimal at 50 °C. The probe was a DNA fragment spanning the 5′-flanking region from position 437 to the *Xba*I site (TCTAGA) at position 949 immediately upstream of the translational start codon (Fig. 5). Following hybridization, 150 μl of a solution containing 200 units/ml S1-nuclease (Boehringer Mannheim, Federal Republic of Germany), 250 mM NaCl, 30 mM sodium acetate (pH 4.5), and 1 mM $ZnCl_2$ was added and the reaction incubated at 37 °C for 45 min. The protected hybrids were ethanol precipitated and analyzed by electrophoresis through 7 M urea, 8% polyacrylamide gels. The gels were exposed to Kodak XAR film with intensifying screens at −70 °C for several days. An M13 sequence ladder, primed with the M13 universal primer, was electrophoresed in parallel to determine the size of the protected fragments.

Polymerase Chain Reactions—Total cytoplasmic RNA (1–2 μg) was subjected to first strand cDNA synthesis in a 20-μl reaction containing 50 mM Tris-Cl, pH 8.3 at 42 °C, 20 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM of each dNTP, 20 μg/ml primer, and 20 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) for 40 min and using as primers either oligo(dT)$_{15}$ or a LIF-specific oligonucleotide (5′-GCCATTGAGCTGTGCCAGTTG-3′) hybridizing to the second exon of the murine LIF gene (positions 2696–2676 in Fig. 5). After completion of first strand synthesis, the reaction was diluted to 100 μl with distilled water and 5 μl used for each PCR reaction. PCR reactions (in a volume of 50 μl) contained 200 μM of each dNTP, 1 μM of each specific primer, buffer as supplied in the GeneAmp kit (Cetus Corp.) and 1.25–2.5 units of Taq polymerase. The reaction conditions were 2 min at 94 °C, 2 min at 65 °C, 3 min at 72 °C for 25 or 35 cycles in a Perkin-Elmer-Cetus DNA thermal cycler. A portion of the PCR reaction was electrophoresed through a 1.3% agarose gel and transferred to nitrocellulose. Filters were prehybridized, hybridized, and washed as described above, but in 6 × SSC. The hybridization probe was an oligonucleotide (5′-GAAAACGGCCTGCATCTAAGG-3′) internal to the amplified fragment (positions 908–928, Fig. 5), radioactively labeled using T4-polynucleotide-kinase and [γ-^{32}P]ATP, radiolabeled to a specific activity of approximately 10^9 cpm/μg.

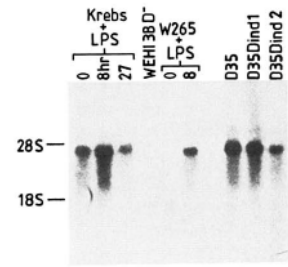

FIG. 1. **Detection of the murine LIF transcript.** RNA from the following sources was probed for LIF transcripts as described under "Materials and Methods": unstimulated Krebs II cells; Krebs II cells cultured at 10^6 cells/ml with 1.5 μg/ml *Escherichia coli* lipopolysaccharide (*LPS*) for 8 or 24 h; WEHI 3BD⁻ cells; unstimulated WEHI 265 cells; WEHI 265 cells cultured at 10^6 cells/ml with 1.5 μg/ml *E. coli* lipopolysaccharide for 8 h; D35, D35Dindl, or D35Dind2 cells (Stocking *et al.*, 1988). The positions of the 28 S and 18 S rRNA molecules are indicated.

RESULTS

The LIF Transcript—Northern blots (such as Fig. 1) revealed that the murine LIF mRNA is approximately 4.2 kb in size, similar to that reported for the human LIF mRNA (Moreau *et al.*, 1988), and is expressed in a variety of cell types, including Krebs II ascites cells, the source from which LIF was originally purified (Hilton *et al.*, 1988b). In Krebs cells and WEHI 265 monocytic cells the abundance of LIF transcripts increased following stimulation with lipopolysaccharide, although in Krebs cells there was a marked basal level which, like GM-CSF (Hilton *et al.*, 1988b), was elevated following stimulation. Other cell types which express LIF mRNA include D35 myeloid cells (Fig. 1), fibroblasts such as L-cells, and the interleukin 2-dependent T-cell clones E9.D4 and LB3 (not shown). We have isolated and sequenced overlapping cDNA clones encompassing most of the murine LIF mRNA, from 22 nucleotides 5′ of the AUG initiation codon, to the end of the 3′-untranslated region (Gearing *et al.*, 1987, 1988, see legend to Fig. 5 for details).[2]

The Murine and Human LIF Genes—In order to provide long range structural information of the LIF gene in murine and human DNA, Southern blots of genomic DNA digested with a series of restriction endonucleases were probed with homologous LIF probes. In human DNA, we have previously reported a unique LIF gene (Sutherland *et al.*, 1989). Similarly in murine DNA, only a unique sequence is detected under conditions of both high and low stringency when a fragment of cDNA corresponding to the coding region is used as a probe (*e.g.* Fig. 2*A* and Gearing *et al.*, 1987). By contrast, a longer probe containing approximately 800 bp of the 3′-untranslated region (pLIFNK3, Gearing *et al.*, 1988) detected additional bands. For example, Fig. 2 shows a Southern blot filter probed under high stringency conditions sequentially with both pLIFmut1 (*A*) and then pLIFNK3 (*B*). It is evident that the latter probe detected multiple sequences, manifest both as dominant bands and as a background smear. While some of these bands represent fragments flanking the LIF coding region, this is not so in all cases. For example, the entire LIF gene lies on a single *Bgl*II, *Hin*dIII, and *Eco*RI fragment (*A*, *tracks 1–3* and see Fig. 3), yet additional fragments became obvious in these digests when probed with the pLIFNK3 probe (*B*).

[2] J. Stahl, D. P. Gearing, T. A. Willson, M. A. Brown, J. A. King, and N. M. Gough, unpublished results.

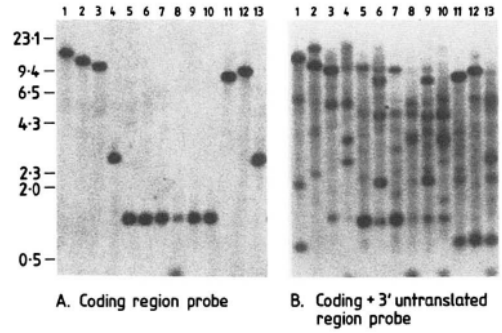

FIG. 2. **Southern blot analysis of the murine LIF gene.** BALB/c liver DNA was digested with the following restriction enzymes: *1*, *Bgl*II; *2*, *Hin*dIII; *3*, *Eco*RI; *4*, *Bam*HI; *5*, *Pst*I; *6*, *Stu*I; *7*, *Pst*I + *Hin*dIII; *8*, *Pst*I + *Bam*HI; *9*, *Stu*I + *Hin*dIII; *10*, *Stu*I + *Bam*HI; *11*, *Bgl*II + *Hin*dIII; *12*, *Bgl*II + *Eco*RI; *13*, *Bgl*II + *Bam*HI. The probe in *panel A* was pLIFmut1 and in *panel B*, pLIFNK3 (see "Materials and Methods" for details). The positions of the λ-*Hin*dIII size markers are shown.

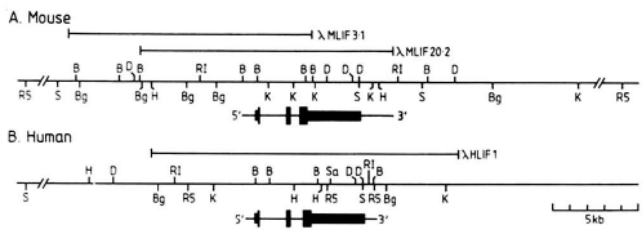

FIG. 3. **Restriction endonuclease cleavage maps of the murine (*A*) and human (*B*) LIF genes.** Data were derived from Southern blots of genomic DNA (*e.g.* Fig. 2*A*, Gearing *et al.*, 1987; Sutherland *et al.*, 1989; and data not shown) and by mapping of LIF genomic clones. The probes used for the murine genomic Southern blots were the cDNA clone pLIFmut1, and an approximately 1-kbp fragment derived from the 3′ end of genomic clone λMLIF20.2 (3′ of the *Hin*dIII site). For the human genomic Southern blots, the 3-kbp genomic *Bam*HI fragment spanning the coding region (Sutherland *et al.*, 1989) was used. The regions of the genome contained in genomic clones λMLIF3.1, λMLIF20.2, and λHLIF1 are shown above the restriction maps. The regions of DNA for which nucleotide sequence were determined (Figs. 5 and 6) and the layout of the LIF genes within these regions are shown below the restriction maps (*large boxes*, coding regions; *medium-sized boxes*, untranslated regions). The murine LIF gene is contained on a single large *Eco*RV fragment (approximately 50 kbp) which spans the map shown. The size of the *Ssp*I fragment extending 5′ of the human LIF gene is approximately 25 kbp, and has been truncated on the map for convenience. Abbreviations: *B*, *Bam*HI; *Bg*, *Bbl*II; *D*, *Dra*I; *H*, *Hin*dIII; *K*, *Kpn*I; *RI*, *Eco*RI; *R5*, *Eco*RV; *S*, *Ssp*I; *Sa*, *Sal*I.

Extensive Southern hybridization data (such as that shown in Fig. 2*A*) allowed the derivation of restriction endonuclease cleavage maps encompassing about 30 kbp of DNA around both the murine and the human LIF genes (Fig. 3).

Cloning and Sequence Analysis of the Murine and Human LIF Genes—The murine and human LIF genes were isolated from λ-genomic libraries by hybridization with a probe corresponding to the murine LIF coding region (pLIFmut1, Gearing *et al.*, 1987; Gough *et al.*, 1988). The clones isolated were subjected to restriction mapping and the regions of the genome contained within each of three λ clones (two mouse, one human) are shown in Fig. 3. The nucleotide sequence of approximately 8.7 and 7.6 kbp encompassing the murine and human genes, respectively, was determined by subcloning appropriate DNA fragments into M13 and pEMBL vectors. Sequencing reactions were initiated with primers located in vector sequences adjacent to the inserts and with primers internal to the subcloned fragments. The direction and extent of sequence determined in each reaction is shown in Fig. 4.

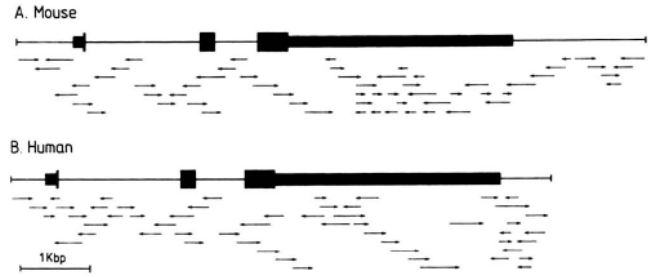

FIG. 4. **Sequencing strategy for the murine and human LIF genes.** *Arrows* depict the direction and extent of nucleotide sequence determined from each primer. Primers were located in both vector sequence adjacent to subcloned DNA fragments and were also located within subcloned DNA fragments. Note that in many instances multiple sequencing reactions and gel readings were obtained with each primer (not shown).

For the most part, sequence was determined from both strands. However in regions for which independent sequence information from cDNA clones was available, sequence was generally only obtained from one DNA strand.

The complete sequences of both genes are displayed in Figs. 5 and 6. The human LIF gene is about 6.3 kbp long, and the murine gene about 6.0 kbp, the difference being due to shorter introns. Both genes consist of three exons and two introns by comparison with their respective cDNA sequences. Exon I encodes the first 6 amino acid residues of the hydrophobic leader, with the remainder encoded by exon II. Exon II also encodes the first 53 residues of the mature protein. The C-terminal 137 amino acids are encoded by exon III, which also specifies an unusually long 3′-untranslated region (3.2 kb). A single poly(A) addition signal (AATAAA) exists at an equivalent position in the murine and human genes (position 6847 in the mouse and 6927 in the human sequence, Figs. 5 and 6, respectively).

A comparison of the sequences of the murine and the human LIF genes reveals that conservation of nucleotide sequences occurs only in certain segments (Fig. 7). Thus, the coding regions of both the murine and human LIF genes are highly homologous (78–94%) and are embedded in non-coding regions which are less conserved. However, a number of short segments within the non-coding regions display strong interspecies homology. In all, there are seven segments including the coding regions, which have homology greater than 75% (Fig. 7). The locations of these regions in each sequence are given in Table I.

Determination of the Transcription Initiation Site—In both genes, there are four "TATA-like" elements in the sequence 300 bp 5′ of the translation initiation codon (indicated in Figs. 5 and 6). In order to determine the start-site of LIF transcription, we employed both an S1-nuclease protection assay and PCR technology. The results of an S1-nuclease assay using cytoplasmic RNA from NIH 3T3 fibroblasts and WEHI3B D$^-$ and WEHI 265 myelomonocytic cells are shown in Fig. 8. A 500-bp DNA fragment extending from an *Xba*I site immediately upstream of the first exon into the 5′ flanking region and encompassing the four TATA elements was used as a probe. In WEHI 265 cells, a 47-bp fragment was found to be protected from S1-nuclease digestion (*lane 2*, Fig. 8*A*) and in WEHI3B D$^-$ cells and NIH 3T3 fibroblasts, a pair of protected fragments at 50 and 51 bp was observed (*lanes 3* and *5*). As the probe was 5′ end-labeled at the *Xba*I site at position 948 (Fig. 5), the sizes of the protected fragments indicate that the start site of LIF transcription is at positions 898/899 in NIH 3T3 and WEHI3B D$^-$ cells and at position 902 in WEHI 265 cells. Similarly a start site at positions

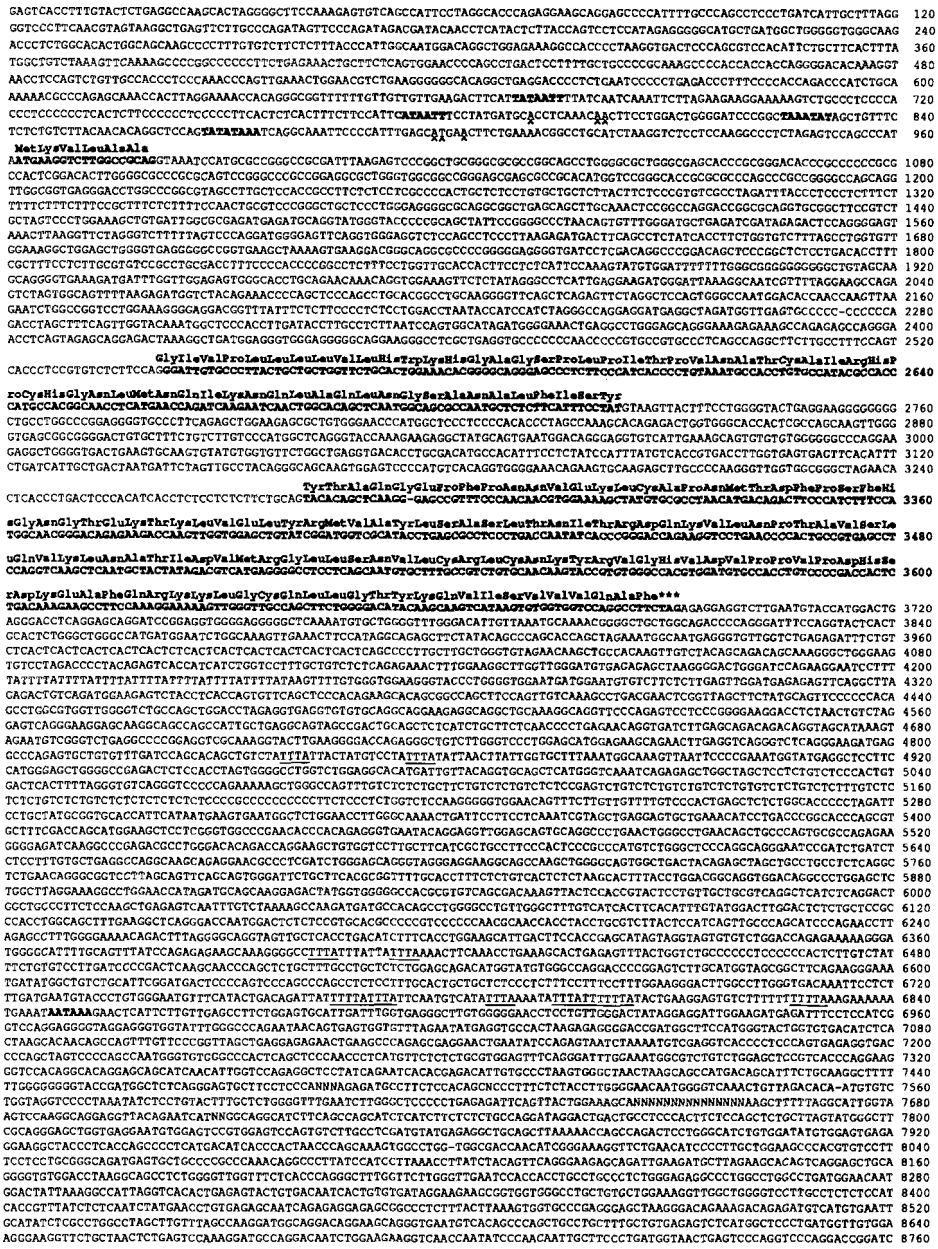

FIG. 5. **Nucleotide sequence of the murine LIF gene.** The layout of exons within the gene was determined both by comparison to the human gene (Fig. 6) and human cDNA sequence (Moreau *et al.*, 1988) and to a set of overlapping murine cDNA clones, pLIF7.2b (Gearing *et al.*, 1987), pLIFNK3 (Gearing *et al.*, 1988), pLIF6a and pLIFJK16.2.[2] Together these cDNA clones encompass most of the LIF mRNA from 22 nucleotides 5′ of the AUG to the 3′ end of the 3′-untranslated region. A region of 776 bp within the 3′-untranslated region (positions 5188–5964) was not included in these cDNAs. The coding regions of the three exons, four TATA-like elements in the region 5′ of the translational start codon and a unique poly(A) signal (AATAAA) are in *bold type*. The start sites of LIF transcription are indicated with *arrows* underneath the sequence line [↞ and ↖, respectively, see text for details). UnA motifs in the 3′-untranslated region that are conserved between the murine and the human LIF genes at equivalent positions are *underlined.*

898/899 has been determined for LIF mRNA from J774 monocytic cells (not shown). These start sites are adjacent to a highly conserved TATA box at position 867 or 869 (Fig. 8*B*).

Since the intensity of signal obtained for S1-nuclease protection experiments was often weak, we employed PCR technology to specifically amplify the 5′ end of the LIF transcript in a modified "primer extension" assay. For PCR experiments, RNA was subjected to first strand cDNA synthesis, and then a set of PCR reactions performed in which each reaction contained the same 3′-oligonucleotide primer (located within exon 2) but a different 5′-oligonucleotide primer which was based on the genomic sequence in the region of the "TATA-like" elements (Fig. 9*A*). Amplification in a PCR reaction will only occur if both primers anneal to their target sequence and hence if the 5′ primer lies within the transcribed region. Initially, a broad region 500 bp upstream of the translational initiation codon was analyzed, and based on these results we focused on the region displayed in Fig. 9*A*. Several experiments were performed with a range of cell types containing LIF mRNA, reverse transcribed using either oligo(dT) or a LIF-specific oligonucleotide as primer. For example, *B* shows the result of an experiment using D35 RNA, in which primers 1 and 2 gave rise to an amplification product (*tracks 1* and *2*), but in which primers 3–6 did not (*tracks 3–6*). In several different experiments using D35 RNA, PCR amplification occurred only with primers 1 and 2, but never with primers 3–6 (see Table II). Identical results were obtained with RNA from NIH 3T3 fibroblasts, WEHI 265, WEHI3B D$^-$, and J774 myelomonocytic cells, in which only primers 1 and 2 resulted in amplification products (Table II). By contrast, Krebs cDNA generally showed amplification also with the primer 3 and, to a lesser extent, with primer 4 (*C*, *tracks 3* and *4*), indicating the presence of slightly longer LIF mRNA molecules in Krebs cells than in D35 cells. As with D35, primers 5 and 6 never gave rise to amplification products in Krebs cDNA (*e.g. tracks 5* and *6* and see Table II). In all RNAs used, there was no significant difference in signals obtained with primers 0, 1, 2, and 3.

Given the high annealing temperature used during the PCR experiments (65 °C), it is likely that a substantial portion of a primer (possibly 10–15 nucleotides of a 20-mer) must bind

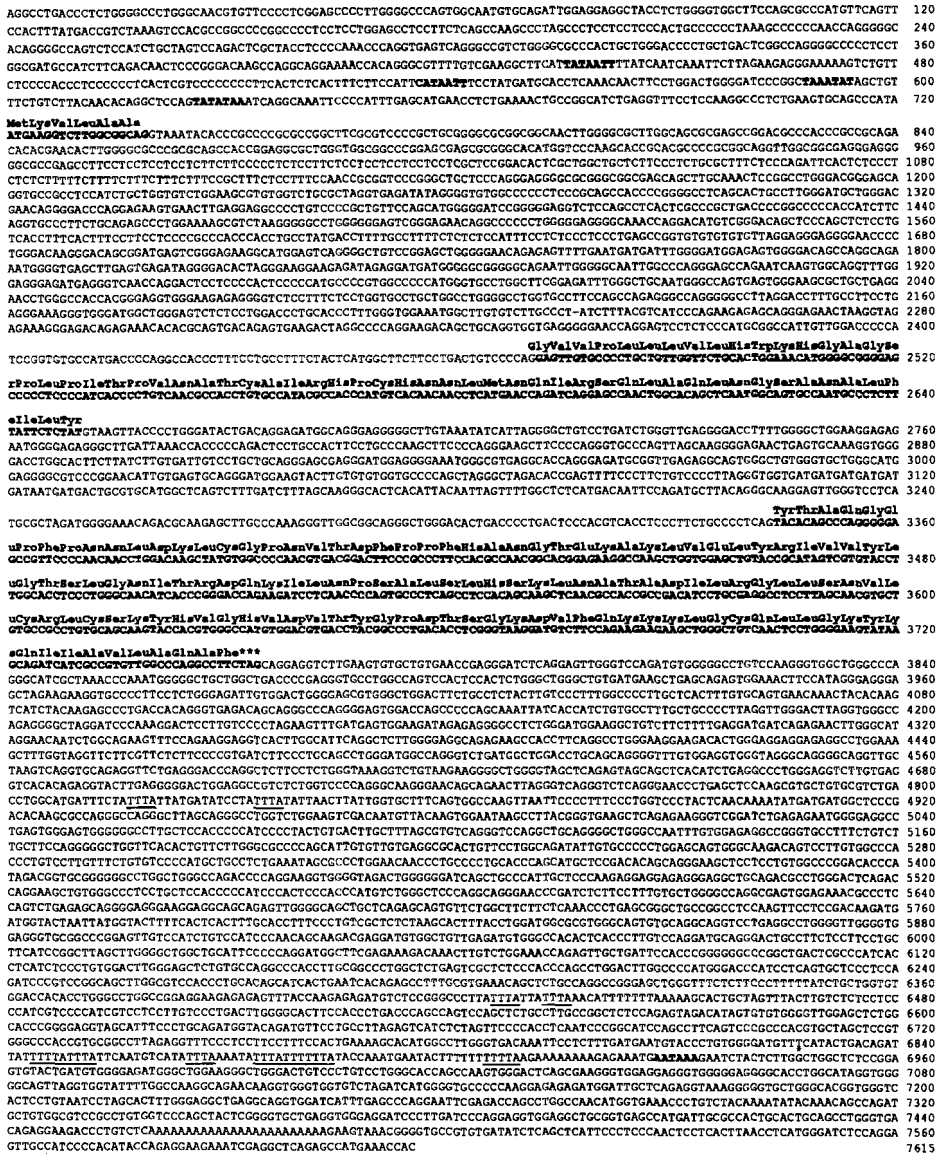

FIG. 6. **Nucleotide sequence of the human LIF gene.** The layout of the exons within the gene was determined by comparison to murine (see Fig. 5 for details) and human (Moreau *et al.*, 1988) cDNA clones. The coding regions of the three exons, four TATA-like elements in the region 5′ of the translational start codon, and a unique poly(A) signal (AATAAA) are in *bold type*. UnA motifs in the 3′-untranslated region that are conserved between the murine and the human LIF genes at equivalent positions are *underlined*. The 3′ end of the human LIF mRNA (Moreau *et al.*, 1988) is indicated by an *asterisk* (*, position 6946).

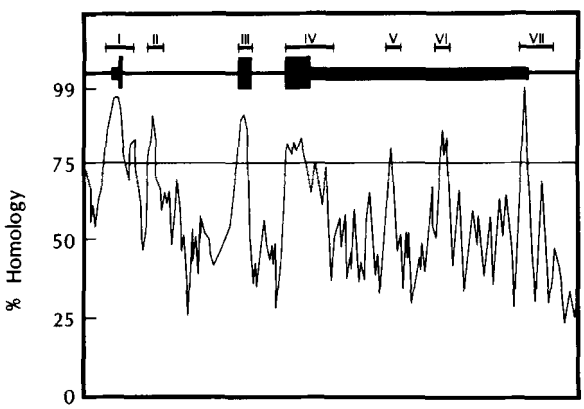

FIG. 7. **Percentage homology plot of the murine and human LIF genes.** The murine and human LIF genes were aligned using the ALINED program (Sidman *et al.*, 1988) with a break penalty of 3 and then percent homology calculated and plotted on a graphic scale (Dr. A. P. Kyne, personal communication) using a window size of 99. Seven segments (*I–VII*) of greater than 75% homology are indicated in *bars* above the layout of the LIF gene (*large bars*, coding regions and *medium-sized* bars, untranslated regions). The locations of these segments in the murine and the human LIF gene sequences are given in Table I.

to the template for amplification to ensue. Thus, in the case of D35 cells, the LIF transcriptional start site must be located 5′ of position 807 (to give at least 10 bp homology to primer 2), but 3′ of position 791 (less than 10 bp homology to primer 3). The distance from this region to the next 5′ located TATA-like element (CATAATT, position 773 in Fig. 5) is between 20 and 35 nucleotides. Applying a consensus sequence for transcriptional start sites associated with TATA boxes (Breathnach and Chambon, 1981), it can be concluded that the start site of LIF transcription defined in these PCR experiments is most likely to be the adenosine residue at positions 800 or 801.

In Krebs cells, the start site of LIF transcription must be located 5′ of position 792 (for primer 3 to give efficient amplification) and 3′ of position 785, and it is therefore possible that the adenosine residue at position 791 would represent the start site of transcription. Some LIF mRNA molecules in Krebs cells, however, appear to be slightly longer, and their transcriptional start site can be mapped between positions 775 and 786.

These results obtained by two different experimental approaches suggest that the transcription of the LIF gene starts at least two different positions. A major start site as determined by S1-nuclease protection assays is between positions

TABLE I

Location of conserved regions within the murine and human LIF-gene sequences

Segments of greater than 75% homology in Fig. 7 are detailed with reference to Figs. 5 and 6. The start of exon I is taken to be the start site of transcription as determined by S1-nuclease protection (see text).

Region in Fig. 7	Genetic region	Position in murine sequence	Position in human sequence
I	5′-Flanking region	653–897	420–656
	Exon I	898–979	657–738
	Intron I	1012–1180	771–934
II	Intron I	1312–1477	1089–1255
III	Exon II	2554–2746	2483–2673
IV	Exon III coding region plus 3′-untranslated region	3139–3890	3204–3954
V	Exon III 3′-untranslated region	4702–4897	4678–4878
VI	Exon III 3′-untranslated region	5496–5722	5462–5685
VII	Exon III 3′-untranslated region	6666–6895	6750–6978
	3′-flanking region	6967–7034	7077–7146

898 and 902, giving a 5′-untranslated region of 60–64 bp. A minor start site was revealed by a more sensitive PCR method and found to occur at nucleotide positions 800 or 801 (and position 791 in Krebs cells), giving a 5′-untranslated region of 160 bp. Both start sites are adjacent to TATA-like elements.

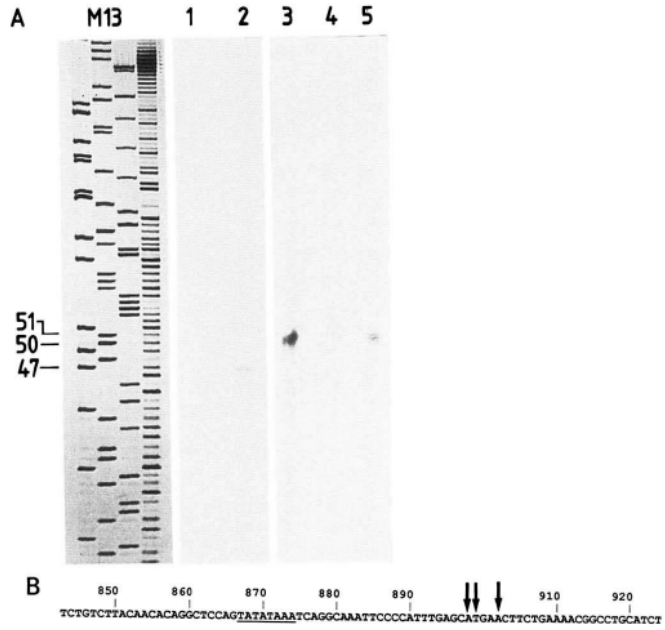

FIG. 8. *A*, S1-nuclease protection assays using mRNA from different cellular sources. A double-stranded DNA fragment encompassing a region of 500 bp upstream of the translational start codon was hybridized at 50 °C with either tRNA as a negative control (*lane 1*) or cytoplasmic RNAs from WEHI 265 cells (*lane 2*), NIH 3T3 cells (*lane 3*), WEHI3B D$^-$ cells (*lane 5*). *Lane 4* shows the result obtained at 53 °C hybridization temperature. An M13 sequence was electrophoresed in parallel to allow size comparisons of the protected fragments. In WEHI 265 cells, a 47-bp band was detected (*lane 2*), whereas in NIH 3T3 and WEHI3B D$^-$ cells a pair of bands at 50 and 51 bp are apparent (*lanes 3* and *5*). *B*, nucleotide sequence in the region of the transcriptional start site of the murine LIF gene as determined by S1-nuclease protection experiments. The *numbers* above the sequence line refer to the numbering system of Fig. 5. The *arrows* indicate the start sites of transcription as determined in part *A*.

DISCUSSION

The biological actions of LIF appear to be diverse and may imply a complex regulation of the LIF gene under different circumstances. Here we have compared the LIF genes from two different species, delineated the LIF transcriptional unit, and identified possible genetic regulatory regions by defining DNA segments with strong interspecies homology. This approach is an initial step in investigating the regulation of LIF production in various cell types.

Organization of the LIF Gene—In view of the biochemical heterogeneity previously reported for different preparations of LIF (Tomida *et al.*, 1984; Koopman and Cotton, 1984; Abe *et al.*, 1986; Godard *et al.*, 1988; Hilton *et al.*, 1988a), we have searched for LIF-related genes by Southern blot hybridization. A complex hybridization pattern, potentially indicative of multiple related genes, became evident when a mouse LIF cDNA containing both coding and 3′-untranslated region sequences was used as a probe (Fig. 2*B*), yet when a probe encompassing only the LIF coding region was applied, a pattern consistent with a unique gene was observed (Fig. 2*A* and see also Gearing *et al.*, 1987). Similarly in human DNA, a unique Southern blot pattern has been noted (Sutherland *et al.*, 1989).[2] Moreover, a unique chromosomal localization has been determined for both murine and human LIF genes (chromosome 11A1-A2, Kola *et al.*, 1990 and 22q12, Sutherland *et al.*, 1989). Thus, it would seem that there are no kindred genes in the murine or human genome which bear significant nucleotide sequence homology to the LIF coding region. It is likely that the additional bands evident in Fig. 2*B* are due to sequences with homology to parts of the 3′-untranslated region of the LIF gene.

A comparison of the murine and human LIF gene sequences reveals that both genes are conserved in their coding regions, but only in parts of the non-coding and flanking regions (Fig. 7). Seven peaks of homology were identified in which the homology between the murine and human LIF genes is greater than 75% (Fig. 7). The highly conserved sequences in the non-coding regions (within *segments I*, *II*, *IV–VII* in Fig. 7) may play a functional role in the regulation of the LIF gene, and thus it will be of interest to compare these sequences with the corresponding regions of the LIF gene from another mammalian species to confirm their conservation.

In general, there is only slight sequence homology between the introns of these two genes, except for two regions within the first intron (*segments I* and *II* in Fig. 7). Evolutionary divergence of intron sequences has been reported for a number of genes such as β-globin, metallothionein I and II, interleukin 2, and G-CSF (Van Ooyen *et al.*, 1979; Searle *et al.*, 1984; Fuse *et al.*, 1984; Tsuchiya *et al.*, 1987). Nevertheless, there are examples of genes with highly conserved sequences in the introns, *e.g.* interleukin 6 and GM-CSF (Tanabe *et al.*, 1988; Miyatake *et al.*, 1985), which have been taken to represent *cis*-acting control elements. Indeed, in contrast to the LIF gene, the gene for interleukin 6 displays its highest degree of interspecies homology in the non-coding regions (Tanabe *et al.*, 1988).

A comparison of the murine LIF gene sequence to the

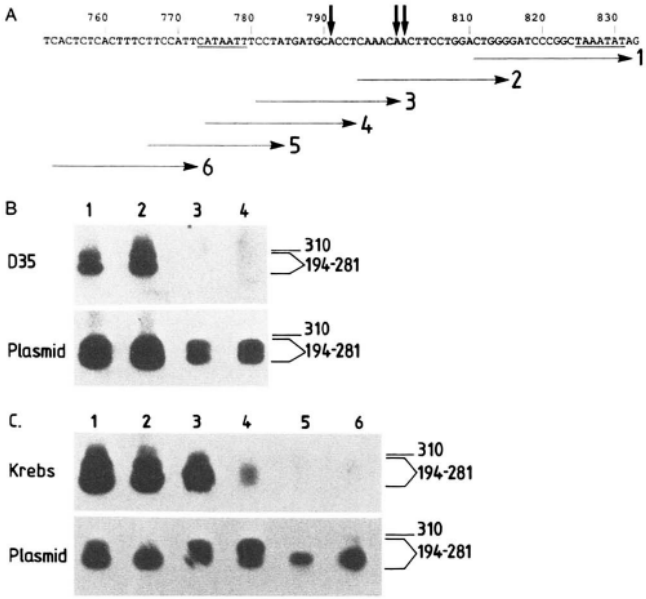

FIG. 9. *A*, nucleotide sequence in the region of the transcriptional start site of the murine LIF gene as determined by PCR. The *numbers* above the sequence line refer to the numbering system of Fig. 5. The location of oligonucleotides used as 5′ primers in PCR reactions is indicated. TATA-like elements are *underlined*. The residues concluded to be the start sites of murine LIF transcription are indicated with *bold arrows* (see text for details). *B*, representative PCR experiment using D35 RNA. *Reactions 1–4* contained 5′ primers 1–4, respectively, in conjunction with a 3′ primer in exon II (at position 2577–2554). A plasmid containing the segment of murine genomic DNA spanning the relevant region was used as a positive control for the PCR reactions. In these latter reactions, the 3′ primer was located in exon I, (979 and 959). Negative control reactions containing D35 or Krebs RNA but omitting reverse transcriptase were performed in parallel and were always negative. The positions of relevant ϕX174-*Hae*III size-markers are shown. *C*, representative PCR experiment using Krebs II ascites RNA. *Reactions 1–6* contained 5′ primers 1–6, respectively, and were as outlined for Fig. 9*B*.

GenBank database (version 58.0) revealed that a long CT-rich repeat in the 3′-untranslated region of the LIF gene (position 5104–5210 in Fig. 5) occurs in a variety of eukaryotic genes, including murine interleukin 1 (Telford *et al.*, 1986), murine interleukin 5 (Campbell *et al.*, 1988), and murine tumor necrosis factor-β (Gray *et al.*, 1987). However, this CT repeat is not contained in the human LIF gene. Such DNA segments consisting of tandem repetitions of two nucleotides have been described as ubiquitous repetitive components of eukaryotic genomes, which probably have no direct function but may form "hot spots" for recombination (Hamada *et al.*, 1982; Tautz and Renz, 1984).

In the 3′ flanking region of the human LIF gene (position *7190–7490*, Fig. 6), there is a 300-bp sequence which shares strong homology (74–85%) with DNA fragments belonging to the Alu family of repetitive sequences (Jelinek and Schmid, 1982; GenBank data-base, version 58.0).

The Promoter Region of the LIF Gene—The transcriptional start site of the murine LIF gene was determined by S1-nuclease protection assay and a novel approach using PCR technology (see Fig. 9 and "Results"). Two different start sites were identified, at positions 898–902 and positions 791–801 (Figs. 8*B* and 9*A*). The transcriptional start site as determined by PCR is thus almost 100 nucleotides upstream of the one identified by the S1-nuclease assay.

To explain these results we suggest that the majority of LIF transcripts initiate at positions 898–902 and could be identified by the S1-nuclease assay. In addition, owing to its high sensitivity, the PCR approach is able to detect a minor subpopulation of transcripts initiating 5′ of this region, at positions 791–801. However, in our hands the PCR approach described here could not be accurately quantified and therefore PCR amplification 3′ of the major transcriptional start site as defined by the S1-nuclease approach (*e.g.* using PCR primer 0 in Table II) gave the same intensity signal as amplification products spanning this site (*e.g.* using PCR primers 1 and 2). Moreover, minor transcripts of intermediate length would also not be detected, for example we cannot rule out the possibility of transcripts initiating adjacent to the TATA box at position 825 (Fig. 5).

Both transcriptional start sites are located 20–30 nucleotides downstream of two of the four TATA boxes present in the sequence. The major start site around positions 898–902 is adjacent to the most conserved TATA box (Breathnach and Chambon, 1981), whereas the initiation site at positions 791–801 is in the proximity of a less well-conserved TATA box within the 5′ region of the LIF gene.

As shown in Fig. 10, there is over 90% homology between the 5′-untranslated and flanking regions of the murine and human LIF genes over a region of some 300 bp 5′ of the translational initiation codon. Such strong conservation of upstream sequences has been described for other genes (Miyatake *et al.*, 1985; Tanabe *et al.*, 1988; Fuse *et al.*, 1984). In fact, some genes (such as GM-CSF, Miyatake *et al.*, 1985) display their highest degree of interspecies homology in the 5′ flanking region, and it appears that this region of the GM-CSF gene contains sequences important for its tissue-specific regulation (Shannon *et al.*, 1988; Nimer *et al.*, 1988). It is likely therefore that sequences important for the regulation of LIF transcription may be contained in this conserved region (Fig. 10).

A pyrimidine-rich region (murine position 712–769 in Fig. 10) contains three repeats of the hexanucleotide "CCTCCC" in the mouse sequence and one such element in the human sequence. Similar elements have been implicated to have enhancer function in some viral genomes (Rosenthal *et al.*, 1983) and have also been shown to be necessary for the function of certain cellular promoters (Johnson *et al.*, 1988; Eaton *et al.*, 1987). It is possible that these elements play a similar role in the LIF gene. Possible binding sites for nuclear proteins in the 5′ flanking region include an "AP-2-like" binding domain in both the murine and the human LIF gene sequences (Fig. 10; Imagawa *et al.*, 1987), and an "SP-1-like" binding domain in the mouse (Dynan and Tjian, 1983) (Fig. 10). It should be noted, however, that this sequence motif is not well conserved in the human LIF gene. A "CAAT" element is present in both the human and murine LIF genes (Fig. 10).

The 3′-untranslated Region—The human and mouse LIF genes have unusually long 3′-untranslated regions (3.2 kb). Although the reasons for such long non-coding regions are unclear, there are other genes, for example the steroid receptors (Misrali *et al.*, 1988; Hollenberg *et al.*, 1985) and basic fibroblast growth factor (Prats *et al.*, 1989), which also have long 3′-untranslated regions. However, in contrast to the LIF gene, these have more than one poly(A) addition signal in their 3′-untranslated regions.

Although the overall homology between the 3′-untranslated region of the mouse and human LIF genes is low, there are blocks of striking homology (Fig. 7). Indeed, the most striking homology between the LIF genes (approximately 100%) is found in a region of 200 bp immediately upstream of the poly(A) addition signal (*peak VII* in Fig. 7). This highly conserved segment might contain sequences involved in LIF mRNA end formation or mRNA stability.

TABLE II

Summary of PCR experiments used to determine the start site of LIF mRNA transcription

The sources of RNA and primers used for cDNA synthesis (LIF-specific oligonucleotide "LIF" (see "Materials and Methods") or oligo(dT_{15})) are given. The 5′ primers used for PCR reactions are indicated (0–9). The locations of primers 1–6 are given in Fig. 9*A*. Primers 0, 7, 8, and 9 were used to initial experiments and their locations in Fig. 5 are 0, 908–927; 7, 693–712; 8, 655–674; 9, 536–550. PCR reactions which gave rise to amplification products are indicated with "+", negative reactions are marked with "−." Low levels of amplification (see Fig. 9*C*) are indicated with (+). W265(−) and W265(+), WEHI 265 cells uninduced (−) and induced (+) with LPS (see "Materials and Methods"). ND, not done.

RNA	Primer used for cDNA synthesis	0	1	2	3	4	5	6	7	8	9
Krebs	Oligo(dT)	+	+	ND	ND	ND	ND	ND	ND	ND	−
Krebs	Oligo(dT)	+	+	ND	ND	ND	ND	−	−	−	−
Krebs	Oligo(dT)	+	+	+	ND	−	ND	−	−	ND	ND
Krebs	LIF	+	+	+	ND	−	ND	−	−	ND	ND
Krebs	LIF	+	+	+	ND	(+)	ND	−	−	ND	ND
Krebs	LIF	ND	+	+	+	(+)	−	−	−	−	ND
Krebs	Oligo(dT)	ND	+	+	−	−	−	ND	ND	ND	ND
D35	LIF	ND	+	+	−	−	−	ND	ND	ND	ND
D35	LIF	ND	+	+	−	−	ND	ND	ND	ND	ND
D35	LIF	ND	+	+	−	−	ND	ND	ND	ND	ND
D35	Oligo(dT)	ND	+	+	−	−	ND	ND	ND	ND	ND
J774	LIF	ND	+	+	−	−	ND	ND	ND	ND	ND
W265(−)	LIF	ND	+	+	−	−	ND	ND	ND	ND	ND
W265(+)	LIF	ND	+	+	−	−	ND	ND	ND	ND	ND
NIH3T	LIF	ND	+	+	−	−	ND	ND	ND	ND	ND
WEHI3BD⁻	LIF	+	+	+	−	−	ND	ND	ND	ND	ND

FIG. 10. **Alignment of the conserved portion at the 5′ end of the murine (*M*) and human (*H*) LIF genes.** Alignment was performed using the ALINED computer program by Sidman *et al.* (1988) with a break penalty of 3. The numbers to the right refer to the sequences in Figs. 5 and 6. Nucleotide identities are indicated by *asterisks*. Breaks introduced into the sequence to maximize homology are indicated by *dots*. The start sites of LIF transcription are shown by *arrows* between the sequence lines (see text and Figs. 8*B* and 9*A* for details). TATA like elements are in *bold type* and *underlined*. Potential binding sites for transcription factors (*AP-2-like*, *SP-1*) and repeats of the hexanucleotide "CCTCCC" (see "Discussion" for details) are *underlined*.

AU-rich sequences of the form "UnA" in 3′-untranslated regions have been inferred to play a role in mRNA stability (Shaw and Kamen, 1986; Caput *et al.*, 1986; Wreschner and Rechavi, 1988; Schuler and Cole, 1988). There are three regions in the LIF gene which contain UnA elements and which might therefore play a role in determining the stability of the LIF mRNA. The most prominent UnA-containing region is between positions 4198 and 4237 in the murine sequence (Fig. 5). This sequence, however, is not found in the human LIF gene suggesting either that the murine and human LIF mRNAs are subject to different regulation or that this sequence does not play a functional role. Segments V and VII of strong interspecies homology (Fig. 7) also contain a small number of UnA sequence motifs which are, however, conserved between mouse and man (*underlined* in Figs. 5 and 6).

Approximately 150 bp downstream of the polyadenylation signal there is a segment consisting mainly of GT-rich repeats (contained in *segment VII* in Fig. 7). Such GT-rich regions have been shown to be required for efficient mRNA 3′ end formation of the rabbit β-globin transcription (Gil and Proudfoot, 1987) and are present in many vertebrate genes (reviewed in Humphrey and Proudfoot, 1988). The "GT-rich" region of the LIF gene may play a comparable role.

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